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EPITOPE MAPPING OF ANTI-YB-1 ANTIBODIES USING TRUNCATED PROTEINS

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ABSTRACT

Increased expression and nuclear localization of Y-box-binding protein-1 (YB-1) are associated with drug resistance and tumor progression. Detection of YB-1 primarily depends on the recognition of YB-1 antibody on its epitopes. To determine the epitopes binding of anti-YB-1 A-16 and 59-Q antibodies, three YB-1 truncated proteins were generated using bacterial expression system and their aberrant migration were reported. Epitope mapping analyses revealed anti-YB-1 A-16 antibody recognized epitope at 1-127 amino acid residues of YB-1, whereas 59-Q antibody was reactive with epitope at 61-127 amino acid residues. Based on our findings, 59-Q antibody was suggested used in the future study to avoid ambiguity.

Key words: epitope mapping, YB-1, truncated protein, antibodies

INTRODUCTION

The multifunctional human Y-box-binding protein-1 (YB-1, P67809), also known as DNA binding protein B (dbpB), belongs to evolutionary conserved cold-shock domain (CSD) family which is widely distributed among bacteria, plants and animals (Wolffe *et al.*, 1992). It plays role in a wide range of cellular functions throughout the cell development (Swamynathan *et al.*, 2002, Fotovati *et al.*, 2011) and survival (Silveira *et al.*, 2012, Lu *et al.*, 2005).

Several reports showed nuclear localization and overexpression of YB-1 are associated with tumor progression and drug resistance (Zhang *et al.*, 2003, Giménez-Bonafé *et al.*, 2004). The multidrug-resistant phenotype of cancers responds to chemotherapy because of the multidrug transporter P-glycoprotein. And this multidrug resistance 1 (*MDR1*) gene encoding P-glycoprotein is probable transcriptionally regulated by YB-1 (Goldsmith *et al.*, 1993, Bargou *et al.*, 1997, Ohga *et al.*, 1998). Thus, YB-1 is suggested as a novel molecular marker of cancer progression and could be a useful target in cancer treatment (Lasham *et al.*, 2013).

A number of anti-YB-1 antibodies have been raised to detect YB-1 protein, including commercially available antibodies or in-house produced antibodies. And these antibodies had been applied to a wide range of human cancers, including cancers of the prostate (Giménez-Bonafé *et al.*, 2004), lung (Shibahara *et al.*, 2001), skin (Schittek *et al.*, 2007), bone (Oda *et al.*, 1998), and others (Chatterjee *et al.*, 2008, Ito *et al.*, 2003, Oda *et al.*, 2003). However, there are evident that different anti-YB-1 antibodies show different immunoreactivity property and are ascribed to the epitopes accessibility and YB-1 protein complexes (Woolley *et al.*, 2011). Therefore, Cohen et al. (2010) warrant re-assessment in some cases about the association of detection of nuclear YB-1 and tumor progression.

The purpose of this work was to map the linear epitopes recognized by commercially available anti-YB-1 antibodies by using truncated proteins. Before this, it is necessary to construct, optimize and purify the production of YB-1 truncated proteins using bacterial expression system. Here, we generated two non-overlapping truncated proteins: N-terminal (N), cold-shock domain (CSD) and one overlapping truncated proteins (N+CSD) for epitope mapping, thus identifying a range of epitopes necessary recognized by anti-YB-1 polyclonal (A-16) and monoclonal (59-Q) antibodies.

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MATERIALS AND METHODS

Construction of truncated genes

The N, CSD and N+CSD gene were amplified from pCR-®2.1-YB-1 plasmid which carries full-length of human *YB-1* gene (1-324 aa), by standard polymerase chain reaction (PCR) using recombinant *pfu* DNA polymerase (Fermentas, USA) with the primers containing *Nde*I and *Age*I cleavage sites. Annealing temperature for each gene was optimized through a gradient PCR. The amplified DNA fragments were purified using QIAquick PCR purification kit (QIAGEN, Germany), and subsequently cloned into pCR®2.1-TOPO® vector (Invitrogen, USA) after adding with 3'A-overhangs using Taq polymerase (Fermentas, USA). *E. coli* strain TOP10 cells was chemically transformed with the plasmids and selected on LB agar plates containing 50 µg/mL kanamycin and 40 mg/mL X-gal. Plasmid DNA of selected cells was purified with QIAprep Miniprep kits (QIAGEN, Germany), then confirmed by restriction analysis using *Nde*I and *Age*I enzymes and verified by M13 forward and reverse DNA sequencing. Then, all DNA fragments in the plasmids were excised at *Nde*I and *Age*I sites and gel purified using QIAquick Gel Extraction kit (QIAGEN, Germany). The purified DNA fragments were ligated to linearized pET16b(SH3) expression vector (Novagen, USA) using T4 DNA Ligase (Fermentas, USA). *E. coli* strain BL21 (DE3) cells were chemically transformed with the plasmids and selected on LB agar plates containing 100 µg/mL ampicillin. Selected cells harboring the plasmid DNA were purified using QIAprep Miniprep kit (QIAGEN, Germany), then confirmed by restriction analysis using *Nde*I and *Age*I enzymes and verified by T7 promoter and terminator DNA sequencing.

Protein Expression and Optimization

E. coli strain BL21 (DE3) cells harboring plasmids were streaked on LB agar plates supplemented 100 µg/mL ampicillin and cultured for overnight at 37ºC. A single colony of bacteria was inoculated into 10 mL LB broth and incubated agitating overnight at 37ºC. The LB medium (10 mL) was added with 100 µL overnight bacterial culture and incubated agitating at 37ºC. At an OD_{600} of ≅ 0.5, 1 mM of IPTG at final concentration was added to induce the protein expression, and then continued incubated agitating and collected at 0 hour, 0.5 hour, 1 hour, 2 hours and 3 hours, respectively. The harvested cells were washed three times with ice-cold PBS (2.7 mM KCl, 137 mM NaCl, pH 7.4) and lysed in urea lysis buffer (7 M urea, 20 mM HEPES, pH 7) added with 3 Unit/mL Benzonase® Nuclease (Merck, Germany). The cells were frozen at -80ºC overnight and vortex vigorously. Supernatant was collected after centrifugation at 9,000 x g for 30 minutes at 4ºC.

SDS-PAGE and Western blotting

Sample proteins were resolved on reducing 12% (w/v) SDS-PAGE and then visualized after coomassie brilliant blue staining. For Western blotting, the gel was transferred to Immobilon P polyvinylidene difluoride filter (Millipore, USA) by semi-dry electroblotting (Biorad, USA) 12 V for 1 h room temperature. The membrane was then blocked with blocking buffer [1x TBS, 5% (w/v) skimmed milk, 0.1% (v/v) Tween 20] for 90 minutes at room temperature under agitating. The membrane was washed three times with washing buffer [0.5% (v/v) Tween 20, 1 x TBS], each for 10 minutes at room temperature. The membrane was then probed with primary antibody at 4ºC for overnight and washed again three times with washing buffer. Then, the membrane was probed with a horseradish peroxidase-linked secondary antibody under agitating at room temperature for 1 hour, followed by three times washing. Image was developed using ECL solution (Millipore, USA) and exposed to Hyperfilm (Amersham, UK) varied for 1-10 minutes, processed using Fuji medical film processor (Fuji, Japan).

Protein purification

A total amount of 6 mL bacterial cleared cell lysate was loaded on 1 mL bed volume of pre-equilibrated nickel-nitrilotriacetic acid agarose (QIAGEN, Germany) and flow through was collected. The column was then washed with 10 mL wash buffer (130 mM imidazaole, 8 M urea, 100 mM NaH_2PO_4, 100 mM Tris-Cl, 300 mM NaCl, pH 8) and wash fraction was collected. Purified protein was eluted 4 times with 500 µL of elution buffer (500 mM imidazole, 8 M urea, 100 mM NaH_2PO_4, 100 mM Tris-Cl, 300 mM NaCl, pH 8) and all fractions were collected.

Antibodies

YB-1 antibodies goat polyclonal (A-16): sc-18057, mouse monoclonal antibody (59-Q): sc-101198, goat anti-mouse antibody sc-2005 and rabbit anti-goat antibody sc-2768 were supplied from Santa Cruz Biotechnology, U.S.A. Anti-6x His tag ® (ab18184) mouse monoclonal antibody [His.H8] was supplied from Abcam, U.S.A.

Cell line

HeLa cells were cultured in DMEM/F12-medium (Invitrogen, U.S.A) containing 10% (v/v) fetal bovine serum (Invitrogen, U.S.A) and harvested after reaching confluent. The harvested cells were subsequently subjected to lysis in RIPA buffer (50 mM TrisCl pH 7.4, 150 mM NaCl, 1% NP40, 0.5%

Na-deoxycholate, 0.1% SDS, 150 mM NaCl, 2 mM EDTA and 50 mM NaF) and incubated overnight at 4ºC. Supernatant was collected after the lysate was centrifuged at 5,000 x g for 30 minutes at 4ºC.

Expression vector

The pET16b(SH3) expression vector, used in this study is shown in figure 2. This modified vector is based on the pET16b expression vector (Novagen, USA) containing 10x histidine residues and added four cysteine residues between *Age*I and *Xho*I cleavage sites, which enable protein-protein interaction study in future.

Protein molecular weight determination

Theoretical molecular weight of proteins was computed using Compute pI/Mw tool from ExPASy (available at: http://web.expasy.org/compute_pi/). The apparent migration size of proteins on 12% (w/v) SDS-PAGE gel was estimated and referred to the pre-stained protein ladder (Fermentas, USA).

RESULTS

Amplification and construction of *YB-1* genes

Amplification of *YB-1* genes (Figure 1) was performed using the designed primers and the optimized parameters listed in Table 1. Our unpublished data demonstrated amplification of *YB-1* genes using these parameters produced good specific target bands by PCR. *YB-1* truncated genes were cloned into the modified pET16b(SH3) vectors (Figure 2) and these constructs expressed 10x histidine tag recombinant proteins and following tailed by 4 x cysteine amino acid residues. Figure 3 showed the His-tagged YB-1 truncated proteins were resolved on 12% (w/v) gel and the membrane by anti-His tag antibody at 1:1000 dilution.

Optimizing expression of YB-1 truncated proteins

Expression of YB-1 truncated proteins in *E. coli* strain BL21(DE3) were optimized through small-scale time-course induction study under induction

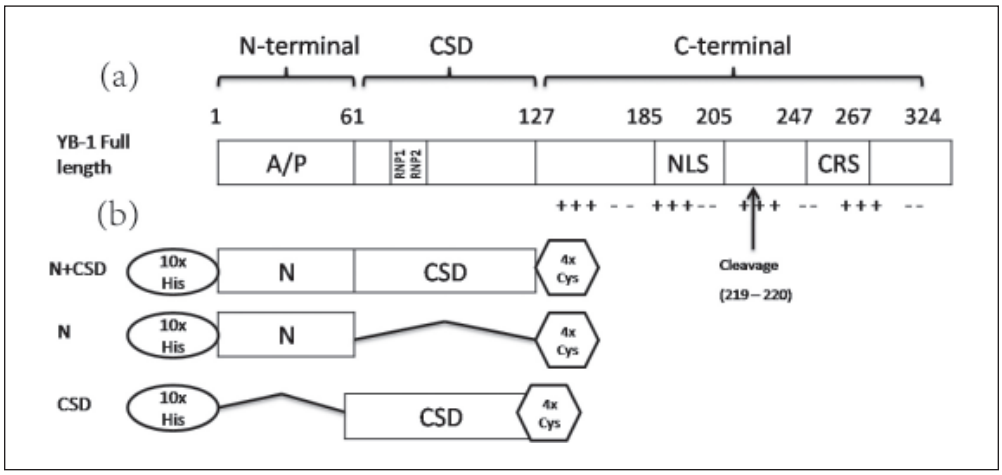

Fig. 1. Schematic illustration of YB-1 full-length protein and truncated proteins. (**a**): Primary structure of YB-1 polypeptide: alanine-proline (A/P) rich N-terminal domain, a central CSD and a tailed C-terminal domain (CTD). CTD consists of a nuclear localization signal (NLS), a cytoplasmic retention signal (CRS) and a 20S Proteasome cleavage site. (**b**): The 10x histidine-tagged YB-1 truncated proteins and tailed by 4x cysteines.

Table 1. Primers for amplification of YB-1 truncated gene. *Nde*I and *Age*I excision sites are indicated in boldface letters for forward primers and reverse primers, respectively

Truncated genes (sizes)	Forward primers	Reversed primers	Optimized annealing temperature (°C)
N (180 bp)	5'-ATG **CAT ATG** AGC AGC GAG GCC GAG -3'	5'-TC**A CCG GT**T TCC CAA AAC CTT CGT TG-3'	55.4
CSD (201 bp)	5'-CGG **CAT ATG** GGA ACA GTA AAA TGG-3'	5'-TC**A CCG GT**G ACC TGT AAC ATT TG -3'	48.0–52.0
N+CSD (381 bp)	5'-ATG **CAT ATG** AGC AGC GAG GCC -3'	5'-TC**A CCG GT**G ACC TGT AAC ATT TG -3'	50.2

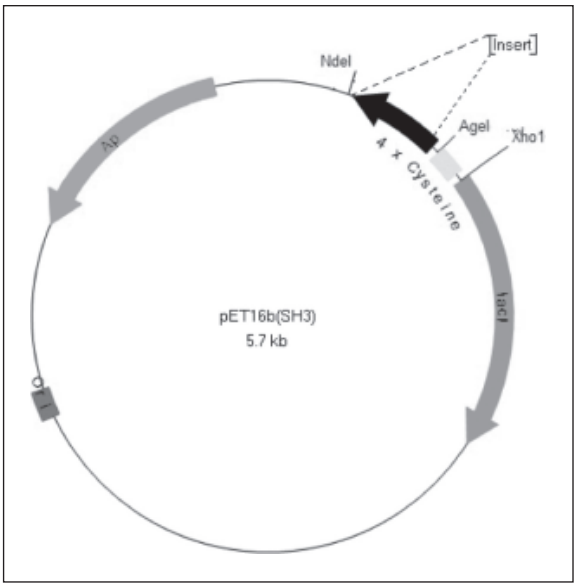

Fig. 2. Plasmid map of modified pET16b(SH3) expression vector (gift of Elena Klenova). It contains the placement of four cysteine amino acid residues at *Age*I and *Xho*I cleavage sites. Digested DNA fragment at *Nde*I and *Age*I cleavage sites can be cloned into pET16(SH3) vector.

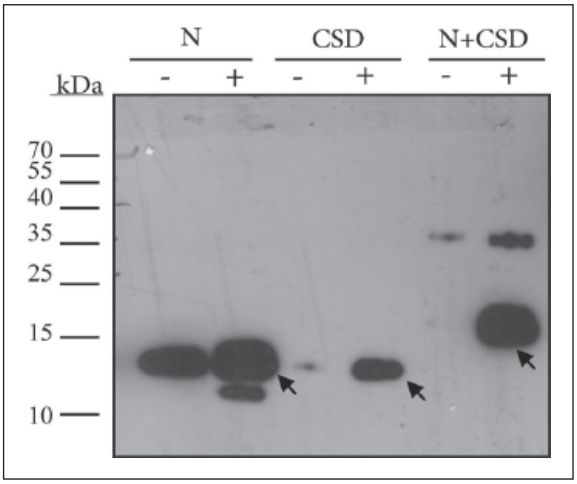

Fig. 3. Expression of His-tagged YB-1 truncated proteins: N, CSD and N+CSD. Cleared cell lysates of expressed proteins were resolved on 12% (w/v) SDS-PAGE and probed by anti-His tag monoclonal antibody on Western blot at 1:1000 dilution. An arrow indicates the authentic protein size. Induction condition of expressed proteins is denoted: uninduced (-) and induced (-).

Table 2. Time-course induction study in *E. coli* strain BL21 (DE3). Expression of YB-1 truncated proteins was induced by 1 mM IPTG at final concentration and harvested at 0, 30 min, 1 hour, 2 hours and 3 hours. Cleared cell lysates containing the truncated proteins were resolved on 12% (w/v) gel and probed by Western blot

Truncated proteins	N	CSD	N+CSD
Optimal induction condition	30 m, 37°C	1 h, 37°C	2 h, 37°C

of 1 mM IPTG at 37ºC. Visual inspection of the apparent thickest band on Western blot probed by anti-His tag antibody was justified as a maximal protein yield. As shown in Table 2, high level expression of truncated proteins was obtained under precise induction timing and temperature.

Optimization and purification of YB-1 truncated proteins

Purification of YB-1 truncated proteins was initially performed through stepwise protein elution study using Ni-NTA spin column (QIAGEN, Germany). Our internal data demonstrated 10x histidine tagged recombinant proteins were starting eluted at 150 mM of imidazole. Thus, YB-1 truncated proteins were washed with 130 mM imidazole and all purified proteins were eluted at 500 mM imidazole buffer. Figure 4-6 showed a single purified protein band were resolved on 12% (w/v) SDS-PAGE.

Anomalous migration of YB-1 truncated proteins

Migration of denatured YB-1 truncated proteins was analyzed on reducing 12% (w/v) SDS-PAGE and probed by Western blot using anti-His tag antibody (Figure 3). The migration size of YB-1 proteins was compared to their theoretical molecular weight and percentage of aberrant migration calculated in Table 3. The percentage of aberrant migration of protein was calculated as following:

$$\%\ of\ Aberrant\ Migration = \frac{SDS\text{-}PAGE\ (kDa) - Theoretical\ (kDa)}{Theoretical\ (kDa)} \times 100\%$$

Validation of anti-His tag antibody and YB-1 antibodies

Prior to Western blot study of YB-1 truncated proteins, anti-His tag mouse monoclonal antibody was validate at 1:1000 dilution, which specifically detected the 70-kDa 10x Histidines tagged zinc fingers of transcriptional repressor CTCF protein (Chernukhin *et al.*, 2000) and was non-reactive to cleared cell lysates of *E. coli* BL21 (DE3) without harboring a plasmid by Western blot (Figure 7a). Anti-YB-1 goat polyclonal (A-16) (sc-18057, Santa Cruz) antibody and mouse monoclonal (59-1) (sc-101198, Santa Cruz) antibody were validated at 1:1000 dilution and specifically detected 49-kDa YB-1 protein in HeLa cell cleared lysate by Western blot (Figure 7b).

Epitope mapping of anti-YB-1 antibodies

Using three purified YB-1 truncated proteins: N, CSD and N+CSD, anti-YB-1 goat polyclonal (A-16) antibody and mouse monoclonal (59Q) antibody were probed on these proteins by Western blot at their validated dilution. Figure 8 demonstrated the

Table 3. The molecular weight determination of YB-1 truncated proteins. Theoretical molecular weight of YB-1 proteins were calculated from ExPASy Compute pI/Mw tool and compared to the apparent migration on 12% (w/v) SDS-PAGE. Their discrepancies in migration are shown in percentage

Truncated protein	Number of amino acids (aa)	Theoretical pI†	Molecular weight/ kDa		Aberrant migration (%)
			Theoretical†	Migration size on 12% (w/v) SDS-PAGE (Reducing)	
N	60	4.94	5.46	14	156.41
CSD	67	5.72	7.81	14	79.26
N+CSD	127	5.82	12.91	22	70.41

†Calculation excluding endogenous pET16b (SH3) vector encoding of 3.18 kDa of 10 x histidine tag and 4 x cysteine tag

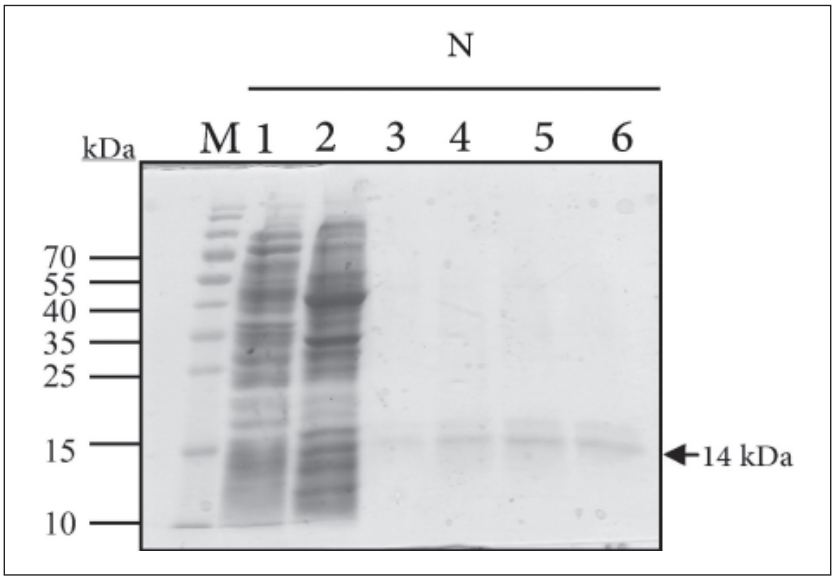

Fig. 4. The purification of N protein was analyzed on 12% (w/v) SDS-PAGE. Lane M: Pre-stained protein ladder; Lane 1: Flow-through fraction; Lane 2: Wash fraction with 130 imidazole buffer; Lane 3-6: Eluted fractions with 500 mM imidazole buffer. Arrow indicates the obtained purified CSD protein.

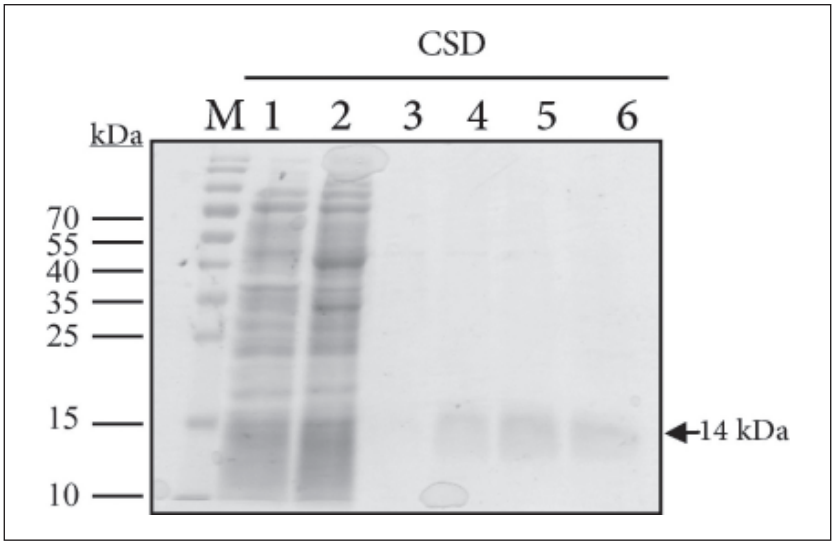

Fig. 5. The purification of CSD protein was analyzed on 12% (w/v) SDS-PAGE. Lane M: Pre-stained protein ladder; Lane 1: Flow-through fraction; Lane 2: Wash fraction with 130 imidazole buffer; Lane 3-6: Eluted fractions with 500 mM imidazole buffer. Arrow indicates the obtained purified CSD protein.

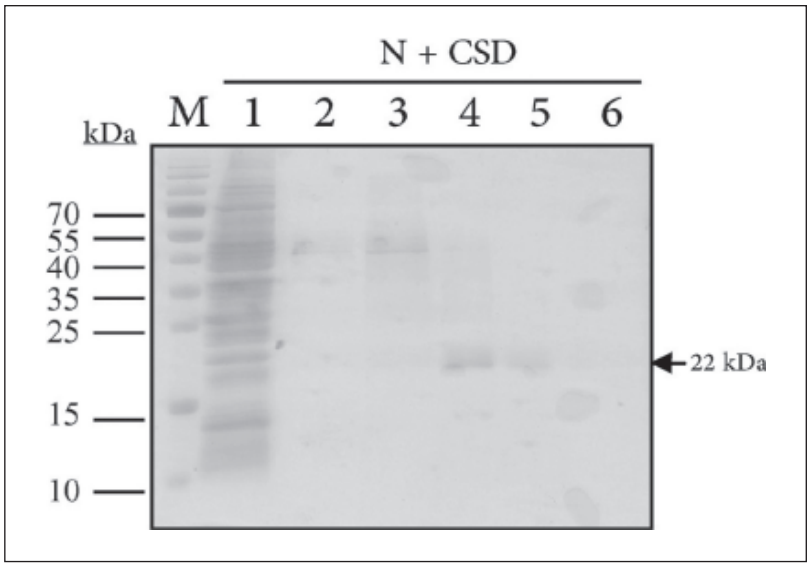

Fig. 6. The purification of N+CSD protein was analyzed on 12% (w/v) SDS-PAGE. Lane M: Pre-stained protein ladder; Lane 1: Flow-through fraction; Lane 2: Wash fraction with 130 imidazole buffer; Lane 3-6: Eluted fractions with 500 mM imidazole buffer. Arrow indicates the obtained purified CSD protein.

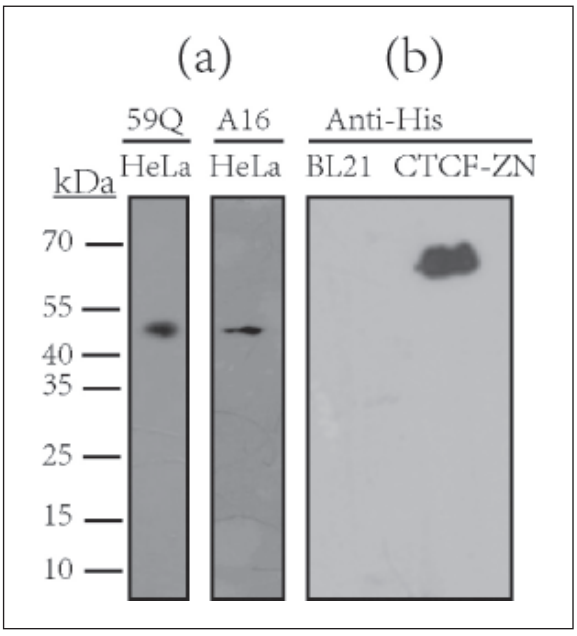

Fig. 7. Validation of anti-YB-1 antibodies and anti-His tag antibody. (**a**) Anti-YB-1 polyclonal antibody (59-Q) and monoclonal antibody (A-16) detected YB-1 protein in HeLa cleared cell lysate in 1:1000 dilution. (**b**) Anti-His tag monoclonal antibody detected zinc fingers of CTCF protein and not reactive with cleared cell lysates of non-harboring plasmid *E. coli* strain BL21 (DE3) in 1:1000 dilution.

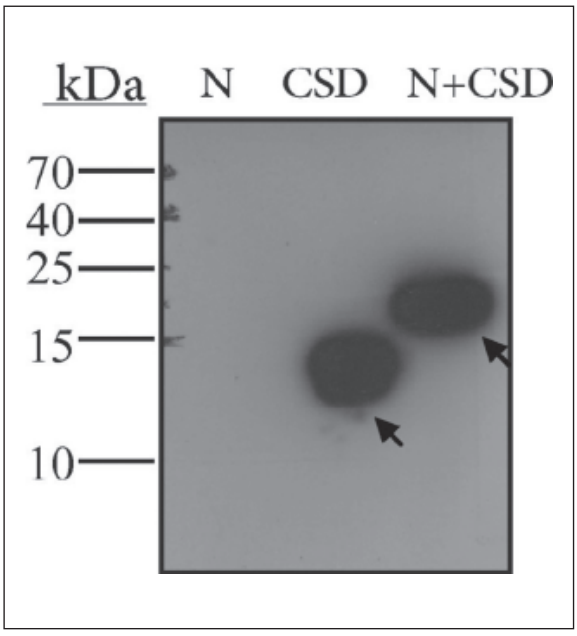

Fig. 8. Western blot of anti-YB-1 (59-Q) monoclonal antibody on YB-1 truncated proteins. These purified truncated proteins were resolved on 12% (w/v) gel and probed by Western blot at 1:1000 dilution of antibody. An arrow indicates the detected protein.

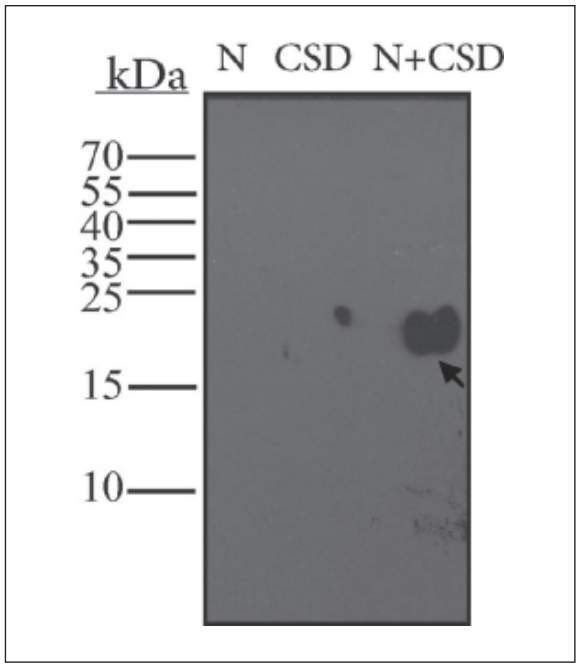

Fig. 9. Western blot of anti-YB-1 (A-16) polyclonal antibody on YB-1 truncated proteins. These purified truncated proteins were resolved on 12% (w/v) gel and probed by Western blot at 1:1000 dilution of antibody. An arrow indicates the detected protein.

Table 4. Summary of epitope mapping of YB-1 antibodies

Anti-YB-1 antibodies	Immunoreactivity		
	N (1-60 aa)	CSD (61-127 aa)	N+CSD (1-127 aa)
A-16 polyclonal antibody	–	–	+
59-Q monoclonal antibody	–	+	+

aa: amino acid residues; (–): not reactive; (+): reactive

bands were detected on CSD protein and N+CSD protein probed by anti-YB-1 (59-Q) mouse monoclonal antibody at 1:1000 dilution. In contrast, Figure 9 indicated only a single band was detected on N+CSD protein probed by anti-YB-1 (A-16) goat polyclonal antibody at 1:1000 dilution. Table 4 summarized the epitope mapping of these anti-YB-1 antibodies.

DISCUSSION

In this study we have shown that the constructed truncated proteins from *YB-1* gene were successfully expressed in *E. coli* strain BL21 (DE3). In addition, these proteins were optimized their expression through the time-course induction study under 1 mM IPTG induction and successfully purified them using immobilized metal affinity chromatography in order to remove bacterial contaminant proteins. In general, YB-1 consists of 324 amino acid residues, the predominating one being Arginine (11.7%), Glycine (12%), Proline (11%), and Glutamine (8.35) (Eliseeva *et al.*, 2011). YB-1 is comprised of three main domains: N-terminal domain (N), Cold-shock domain (CSD) and C-terminal domain (C) (Wolffe, 1994). Here, the denatured YB-1 truncated proteins: N, CSD and N+CSD aberrantly migration on 12% (w/v) reducing gels, despite of their calculated molecular weight. Among these proteins, N protein showed the highest percentage in peculiarity of migration. These aberrant migrations can be as a result of several factors (Rath *et al.*, 2009, Armstrong and Roman, 1993, Manning and Colon, 2004). Here, we speculated N and CSD proteins contributed to anomalous migration of full-length YB-1. However, the protein sequencing of proteins needs to be carried out in order to confirm these results.

Anomalous migration of YB-1 is known to migrate as a 45-50 kDa protein on SDS-PAGE gels; however its molecular weight calculated by the amino acid sequence is about 35.9 kDa. And this discrepancy is suggested due to anomalous electrophoretic mobility (Skabkin *et al.*, 2004). Our results showed using anti-YB-1 (A-16) polyclonal and (59-Q) monoclonal antibodies, migration size of YB-1 protein in HeLa cell lysates on 12% (w/v) reducing gels was identical with the detection of purified p50 (YB-1) from reticulocytes (Evdokimova *et al.*, 1995). In addition, these two antibodies detected no so-called smaller protein band, which was previously presumed as cleavage of a short C-terminal YB-1 fragment by 20S Proteasome (Sorokin *et al.*, 2005) but was then revealed as hnRNPA1 instead (Cohen *et al.*, 2010).

Epitope mapping is the process of identification of the binding sites or epitopes of antibody. A number of methods have been developed for mapping protein epitopes of antibodies (Ladner, 2007). A popular strategy involves probing the antibody-binding ability of synthetic peptides derived from the amino acid sequence of the antigen (Baerga-Ortiz *et al.*, 2002). In contrast, epitope mapping of anti-YB-1 antibodies employed in this study was using YB-1 truncated proteins (linear epitopes) instead of synthetic peptides and it would be able roughly locating the binding sites of antibodies. In fact, this technique had been applied in several epitope mapping studies (Wade-Evans and Jenkins, 1985, Raux *et al.*, 1997, Meysick *et al.*, 2001).

Our data presented anti-YB-1 (A-16) polyclonal antibody recognized YB-1 protein containing epitopes located between 1-127 amino acid residues. Indirectly, this data also implied that N or CSD

protein individually abolished the recognition by anti-YB-1 (A-16) antibody. This 127-amino-acid region belongs to N and CSD domains of YB-1. The N-terminal sequence of YB-1, containing the alanine-proline rich domain, involves in regulating the progression of cells from G2/M to G1 phase in a cell cycle (Khandelwal *et al.*, 2009). While the CSD is an oligosaccharide-/oligonucleotide-binding (OB fold), which comprises of a five-stranded β-barrel that includes two RNA-binding sequence motifs RNP1 and RNP2 (Kloks *et al.*, 2002), and shares functional properties with the major proteins of heterogeneous nuclear RNPs (hnRNPs). Although there is no apparent similarity in amino acid sequences between YB-1 and hnRNPs, but the molecular mimicry between them leads to the cross-reactivity of anti-YB-1 antibody, which recognized the epitope at 23-52 amino acid residues (Cohen *et al.*, 2010). Coincidently, the epitope of anti-YB-1 (A-16) antibody were revealed located at 25-75 amino acid residues (Santa Cruz, personal communication). According to its product datasheet (sc-18057, Santa Cruz), anti-YB-1 (A-16) antibody specifically detected about 37-kDa protein band in several cell lines by Western blot, but we detected at 49 kDa in HeLa cells. In Cohen *et al.* (2010) study, the 37-kDa protein band proposed as activated YB-1 was eventually identified as as hnRNP A1. Thus, it was speculated used of this antibody would probable render the cross-reaction problem and misidentification of YB-1 in several cited studies (Perez-Leal *et al.*, 2012, Sano *et al.*, 2008, Chibi *et al.*, 2008, Homer *et al.*, 2005, Kojic *et al.*, 2004).

On the other hand, anti-YB-1 (59-Q) monoclonal antibody was shown specifically reactive with YB-1 truncated protein containing epitope 61-127 amino acid residues. Therefore, CSD or N+CSD protein containing this epitope region was detected in Western blot except for N protein. In light of this epitope, anti-YB-1 (59-Q) antibody was disclosure against the epitope at 51-140 amino acid residues of YB-1 (Santa Cruz, personal communication), which was approximately identical to our data. Thus, this antibody is recommended to probe the YB-1 protein in future with disambiguation. Taken together these data, it was important to avoid the used of cross-reactivity epitope of amino acid sequence in production of antibody.

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